

Genetic characterization of *Acipenser sturio* L., 1758 in relation to other sturgeon species using satellite DNA

M. Ruiz-Rejón, R. de la Herrán, C. Ruiz-Rejón and M. A. Garrido-Ramos

Departamento de Genética, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain. E-mail: mrejon@ugr.es

Received September 1999. Accepted June 2000.

ABSTRACT

We obtained and characterized a satellite (st) DNA family named HindIII from the genomes of the Adriatic sturgeon *Acipenser naccarii* Bonaparte, 1836, Siberian sturgeon *Acipenser baerii* Brandt, 1869, and beluga sturgeon *Huso huso* (L., 1758). We did not find this stDNA in the genome of the Atlantic sturgeon *Acipenser sturio* L., 1758. The comparison of sturgeon species using the HindIII stDNA revealed the following: (1) *A. naccarii* and *A. baerii* are closely related; (2) *H. huso* appears to belong to the genus *Acipenser* and, probably, *Huso* is not a separate genus within the Acipenserinae; (3) *A. sturio* differs from the other three studied species by the absence of the HindIII stDNA and, most likely, it represents a separate evolutionary lineage within the Acipenseridae. The data on the HindIII stDNA can be successfully used for species identification of sturgeon specimens captured in different European regions.

Key words: Sturgeons, nuclear DNA, phylogenetic relationships, genetic identification.

RESUMEN

Caracterización genética de *Acipenser sturio* L., 1758 con relación a otras especies de esturión por medio de ADN satélite

En este trabajo presentamos la caracterización del genoma de *Acipenser sturio* L., 1758 en relación con el genoma de *Acipenser naccarii* Bonaparte, 1836, *Acipenser baerii* Brandt, 1869 y *Huso huso* (L., 1758) utilizando una familia de ADN satélite (la familia HindIII). Nuestro análisis revela que: (1) *A. naccarii* y *A. baerii* son especies muy emparentadas; (2) *H. huso* aparece muy relacionada con las especies del género *Acipenser* y, probablemente, podría ser considerada como una especie perteneciente a dicho género, y (3) *A. sturio* difiere del resto de las especies analizadas, lo que sugiere que esta especie ha debido seguir una evolución independiente respecto a las otras especies. Estos datos pueden ser muy útiles, no sólo para establecer las relaciones filogenéticas entre *A. sturio* y las otras especies de Acipenseridae, sino también para la identificación de ejemplares de esturiones capturados en diferentes regiones europeas.

Palabras clave: Esturiones, ADN nuclear, relaciones filogenéticas, identificación genética.

INTRODUCTION

Acipenser sturio L., 1758 is a sturgeon species of interest from conservation and evolutionary perspectives. It is considered an endangered species,

for which recovery plans are needed (Birstein, 1993). However, recovery plans can be complicated by the fact that in some parts of the *A. sturio* area it is possible to find some other natural or introduced congeneric species (Rochard *et al.*, 1991).

Thus, historically *A. sturio* was sympatric with the Adriatic sturgeon *Acipenser naccarii* Bonaparte, 1836 and the beluga sturgeon *Huso huso* (L., 1758) in the Adriatic Sea basin (Rossi *et al.*, 1991). Of these species, *A. sturio* has been traditionally included in the genus *Acipenser*, while the beluga sturgeon *H. huso* and its close relative the kaluga sturgeon *Huso dauricus* (Georgi, 1775) were considered as representatives of a separate genus *Huso* within the subfamily Acipenserinae of the family Acipenseridae (Berg, 1904; for details, see Bemis, Findeis and Grande, 1997). However, recent molecular data (studies of mitochondrial DNA) indicate that *Huso* is not a separate genus within the Acipenserinae (Birstein, Hanner and DeSalle, 1997; Birstein and DeSalle, 1998). As for the introduced species, individuals of the non-indigenous Siberian sturgeon *Acipenser baerii* Brandt, 1869 caught in the rivers of Spain (e.g. Birstein, Betts and DeSalle, 1998) were escapes from aquacultured stocks.

In the present study we used a satellite (st) DNA family named HindIII in order to clarify the identification of and to explore the genetic relationships between *A. sturio* and *A. naccarii*, *A. baerii* and *H. huso*. stDNA consists of families of short, tandemly repeated sequences which are located in certain regions of chromosomes, mainly centromeres and/or telomeres. Because of their rapid evolution, the repeated sequences, including stDNA, are frequently used for taxonomic and phylogenetic purposes (Arnason, 1990; Mikhailova *et al.*, 1995; Garrido-Ramos *et al.*, 1999) and for species identification (e.g. Meredith *et al.*, 1991). The comparison of the HindIII stDNA from four sturgeon species showed the close relatedness of *A. baerii*, *A. naccarii*, and *H. huso*, and their distant relatedness to *A. sturio*.

MATERIALS AND METHODS

Sturgeon specimens were obtained from the Sierra Nevada S. L. Fish Farm at Riofrío, Granada, Spain (*A. naccarii* and *A. baerii*), from Azienda Agricola VIP, Brescia, Italy (*H. huso*), and from France's National Agricultural and Environmental Engineering Research Centre (Cemagref) in Bordeaux (*A. sturio*).

DNA was isolated from the liver and muscles of three individuals for each species following the

method of Sambrook, Fritsch and Maniatis (1989). In summary, 1 g of liver from each specimen was ground with liquid nitrogen in a mortar into a powder. After the evaporation of the nitrogen, 10 volumes of the extraction buffer (10 mM Tris-HCl plus 0.1 M EDTA, pH = 8), 20 µg/ml pancreatic RNAase and 0.5 % SDS were added. The solution was transferred to a 30 ml centrifuge tube and incubated for 1 h at 37 °C. Thereafter, Proteinase K was added to a final concentration of 100 µg/ml and incubation was performed for 3 hours at 50 °C. The DNA obtained was purified using the common phenol/chloroform procedure and, finally, precipitated with ethanol. The pellet of DNA was then dissolved in the TE buffer (10 mM Tris-HCl plus 1 mM EDTA, pH = 8).

The HindIII sequences in the genomes of different sturgeon species were detected by Southern-blot hybridization. The hybridization and the detection of the hybridization sites were performed using the non-radioactive chemiluminescence method, ECL (Amersham), following the manufacturer's instructions. Filters were hybridized for 12-16 h at 42 °C with a peroxidase-labeled probe at 10 ng/ml of the ECL hybridization buffer containing 6 M urea, 0.5 M NaCl, and 5 % blocking agent. After hybridization, the membranes were washed twice, for 20 min each, in 6 M urea, 0.1 X SSC and 0.4 % SDS at 42 °C (high-stringency conditions) or 1 X SSC and 0.4 % SDS at 55 °C for 10 min (low-stringency conditions). The membranes were then washed twice in 2 X SSC at room temperature for 5 min. The 6 M urea in the hybridization and wash buffer is equivalent to 50 % formamide (Amersham). Hybridization sites were detected using the ECL detection reagents, which contained hydrogen peroxide and luminol, and the light emitted in the detection reaction was recorded radiographically.

To clone the HindIII stDNA from different species that have the HindIII sequences, samples of DNA obtained from these species were digested with HindIII restriction enzymes, electrophoresed on a 3 % NuSieve agarose gel, and stained with ethidium bromide. Intense DNA bands corresponding to the monomeric HindIII units were excised from the gel and the DNA was purified with phenol extraction. The HindIII units were cloned in the pUC19 plasmid vector. The ligation was used to transform *Escherichia coli* DH5-α competent cells, and bacteria clones containing recombinant

plasmids with the repetitive fragment of interest were selected after screening with an aliquot of labelled DNA from the same band used for cloning. Eleven clones from *A. naccarii*, nine from *A. baerii*, and seven from *H. huso* were selected for sequencing.

Recombinant plasmids containing cloned stDNA sequences were used as templates for sequencing by the dideoxynucleotide chain terminator method of Sanger, Nicklen and Coulson (1977). Sequencing products were analysed using an automated laser fluorescent DNA sequencer (Pharmacia).

Sequence analyses were performed using the computer program GENEPRO v. 6.1 (Riverside Scientific Ent., 1993). For the HindIII sequences, interspecific analysis was studied using method II of Strachan, Webb, and Dover (1985). This method measures the mean variability per nucleotide site using all possible combinations between the different clones of the two species being compared. Basically, this calculation was made in a similar way to the calculation of nucleotide diversity (Nei, 1987) –i.e., adding together all the nucleotide differences between each pair of sequences being compared, and dividing this sum by the number of comparisons made.

RESULTS AND DISCUSSION

First, we analysed a stDNA family named HindIII present in the genome of *A. naccarii*. Our previous study had shown that this stDNA is absent from the genome of *A. sturio* (Garrido-Ramos *et al.*, 1997). Our new results confirm this conclusion: Southern-blot hybridization, using both low and high stringency conditions, did not detect the Hind III stDNA in *A. sturio*. However, we showed that the HindIII stDNA sequences are present in the genomes of *A. baerii* and *H. huso*.

Secondly, we cloned and sequenced several monomeric units of the HindIII stDNA family isolated from the genomes of *A. baerii* and *H. huso* (figure 1). Previously, the cytogenetic experiments (*in situ* hybridization) showed that this stDNA is located in the centromeres of several chromosomes of *A. naccarii* (Garrido-Ramos *et al.*, 1995). Later, Fontana *et al.* (pers. comm.) showed that the HindIII stDNA is also present in the centromeres of several chromosomes of *A. baerii*, the Russian sturgeon *Acipenser gueldenstaedtii* Brandt, 1833, the American white sturgeon *Acipenser transmontanus* Richardson, 1836, and *H. huso*. In contrast, the *A. naccarii* HindIII stDNA did not hybridize with chromosomes of *A. sturio*.

<i>A. naccarii</i>	CTTTTTCAAA	AGC C CTTTTGGGGC	ATTGAAATTA	TGAAAAATA	AAATTGGCCA	AAATTATTAT	TTTTT*TGAC
<i>A. baerii</i>	-----	CT TA AG--CG----	-----	-----	-----	-----	-----
<i>H. huso</i>	----C----	A AG--CG-TA-	-----	-----	-----	-----	----TC--
<i>A. naccarii</i>	AGGACCGGAC	CAGACCACTT	TTTCAAAAAA	GGGGGATGTC	TAAATTTTGG	TAGTTCTGAA	GATCAAAAAA
<i>A. baerii</i>	-----	---T-----	-----	-----*	-----	-----	-----
<i>H. huso</i>	---T-----	---T-----	----G----	-----* ---T-G--	-----G--	-----T----	-----
<i>A. naccarii</i>	TTGTGTTTTTC	TTGACAGGAA	CGAACCTGTA	AG			
<i>A. baerii</i>	-----	-----	-----	--			
<i>H. huso</i>	-----	-----T-TC	T-----	--			

Figure 1. Nucleotide consensus sequences of the HindIII stDNA family in *A. naccarii*, *A. baerii*, and *H. huso*. A consensus sequence for each species was determined by comparative analysis of monomeric sequences isolated from each of the species: 11 monomers for *A. naccarii*, 9 monomers for *A. baerii*, and 7 monomers for *H. huso* were sequenced and analysed. The complete consensus sequence of *A. naccarii* is presented and, in each species, only the differences with respect to the first species are indicated. In variable positions the most frequently occurrent nucleotides are shown. If two nucleotides were equally frequent, the second is given above the consensus sequence. Asterisks indicate deletions

These data can be discussed in phylogenetic terms. The presence of the HindIII stDNA in four *Acipenser* species (*A. naccarii*, *A. baerii*, *A. gueldenstaedtii*, and *A. transmontanus*) and in *H. huso* supports the view of Birstein and DeSalle (Birstein, Hanner and DeSalle, 1997; Birstein and DeSalle, 1998) that the genus *Huso* is in fact not separated from *Acipenser*, and the doubts of Artyukhin (1995) concerning the systematic position of *Huso*. Therefore, *H. huso* appears to belong to the genus *Acipenser*. Additionally, the absence of the *A. naccarii* HindIII sequences from the genome of *A. sturio* supports the hypothesis that *A. sturio* (together with the American Atlantic sturgeon *Acipenser oxyrinchus* Mitchill, 1815) represents a separate evolutionary lineage within the family Acipenseridae (Birstein and Bemis, 1997; Birstein and DeSalle, 1998).

Our analysis revealed that the HindIII monomers in *A. naccarii*, *A. baerii* and *H. huso* are very similar. However, the slight differences in sequences that we found may be valuable evidence for determining the relationships among these species. In *A. naccarii* and *A. baerii*, the mean interspecific sequence divergence between the HindIII repeats was only 2.3 %. However, we found that the mean interspecific sequence divergence between *H. huso* and *A. baerii* HindIII monomers is 8.7 %, and between *H. huso* and *A. naccarii* it is even higher, 11.6 %. Therefore, in *A. naccarii*, and *A. baerii* the HindIII sequences clearly diverge from those in *H. huso*.

These HindIII sequences can be used for the genetic identification of specimens captured in the rivers of western Europe. Previously, when we could find the HindIII stDNA in the DNA extracted from a museum specimen (EBD 8173, the museum collection of the Doñana Biological Station, Spain), we concluded that the specimen was not *A. sturio* (Garrido-Ramos *et al.*, 1997); this specimen

had been captured in the Guadalquivir River (Spain) in 1974 and was initially identified as such. Notwithstanding, the presence of HindIII stDNA in a specimen should indicate that it belongs to one of the species having the HindIII stDNA (i.e., *A. naccarii*, *A. baerii*, or *H. huso*). For species identification, it would be necessary to obtain the stDNA from each specimen, sequence it, and compare it with the diagnostic nucleotide sites given in table I. Some sites (nos. 11, 12, 15, 67, and especially 106) provide an opportunity to differentiate *H. huso* from *A. naccarii*/*A. baerii*, and there are a few differences between the latter two species (sites nos. 10, 67, 87, and 106). Although some of the sites vary intraspecifically (table I), they can be used for species identification.

For example, for the identification of the EBD 8173 specimen, Garrido-Ramos *et al.* (1997) cloned and sequenced a HindIII monomeric unit named pA11 (EMBL accession number Z50744). Although all three species (*A. naccarii*, *A. baerii*, and *H. huso*) have A in position 10, there are some monomer variants of *A. naccarii* in which the nucleotide T replaces A, and the only clone sequenced from the specimen EBD 8173 had T at this site, showing that it corresponds to the *A. naccarii* sequence. Similar reasoning can be used for the rest of sites shown in table I. Based on the sequence analysis of the HindIII monomeric units, we concluded that the EBD 8173 is an *A. naccarii* specimen (table I).

In a recently published paper (Doukakis *et al.*, 2000), three independent laboratories could not confirm our previous molecular identification of the two museum specimens, EBD 8173 and EBD 8174 from the collection of the Doñana Biological Station. The authors could not extract authentic DNA from these samples, and concluded that the DNA extracted and then cloned from the specimen EBD 8173 might have been a contaminant. Later

Table I. Diagnostic nucleotides in the HindIII stDNA sequences of *H. huso*, *A. baerii*, *A. naccarii*, and in the sequence from the specimen EBD 8173. Asterisks indicate a deletion at the site. At variable sites in which two possible nucleotides were found, the more frequent nucleotide is presented in upper case and the less frequent in lower case

Species	Nucleotide position						
	10	11	12	15	67	87	106
<i>Huso huso</i>	A	A	G	C	C	G	T/*
<i>A. baerii</i>	A	A/c	G/t	C/t	C/t	G	*/A
<i>A. naccarii</i>	A/t	C/a	T/g	T/c	T/*	G/t	A/c
Specimen EBD 8173	T	C	T	T	*	T	C

some of the co-authors of this paper (Almodóvar, Machordom and Suárez, 2000) managed to obtain DNA from the specimen EBD 8174 and identified the specimen as *A. sturio*. Be that as it may, we believe that we escaped some technical problems discussed in Doukakis *et al.* (2000): these authors used the PCR technique, and they could have amplified contaminants. This was the reason why, in our study, we used direct cloning of DNA extracts, which obviates the problems of minute contamination with the DNA from other sturgeon species.¹

Finally, it is important to mention that, following an independent technique, comparative erythrocytic cytometry, it has been possible to show (Hernando *et al.*, 1999a, b) that specimen EBD-8173 is a multichromosomal sturgeon with approx. 240 chromosomes (as is well known, *A. sturio* is a species with approx. 120 chromosomes, while *A. naccarii* have approx. 240 chromosomes)².

CONCLUSIONS

Using the HindIII stDNA as molecular markers for studying the relationships among sturgeon species and for genetic species identification, we have shown the following:

- 1) *A. naccarii* and *A. baerii* seem to be closely related; the sequence divergence for the HindIII sequences between these species is only 2.3 %. Previously, the close relatedness of these species had been reported by Birstein (1999), Birstein, Doukakis and DeSalle (2000), and Tagliavini *et al.* (1999) using mitochondrial DNA. It was also described by Artyukhin (1995), on the basis of morphological traits.
- 2) *H. huso* is one of the *Acipenser* species which might be closely related to *A. naccarii* and *A. baerii*. As mentioned above, the fact that two *Huso* species are embedded within *Acipenser* was previously shown by Birstein and DeSalle (Birstein, Hanner and DeSalle, 1997; Birstein and DeSalle, 1998).
- 3) *Acipenser sturio* differs from the other three sturgeon species studied and, most likely, it belongs to a separate evolutionary lineage within the Acipenseridae. These data agree with the results of Birstein and DeSalle (1998) obtained using mitochondrial DNA.
- 4) HindIII stDNA can be a useful tool for molecular species identification of unknown specimens of sturgeons captured in different European regions.

¹ Unfortunately, the authors of the paper have still failed to present serious proof that the DNA they extracted from specimen EBD 8173 (Garrido-Ramos *et al.*, 1997) was authentic, and not a contaminant. Also, they did not take into consideration the new morphology re-examination of specimens EBD 8173 and EBD 8174 discussed by Elvira and Almodóvar (1999; 2000) and Rincón (2000) (these papers are cited in Birstein and Doukakis, 2000). But it seems that the authors no longer insist that specimen EBD 8174 is not *A. sturio*, but *A. naccarii*, as they did in Garrido-Ramos *et al.* (1997) (co-editor's note; July 2000).

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² Both cited papers by Hernando *et al.* (1999a, b) do not contain any details on measurements of erythrocytes and any result of the measurements. The size comparison of erythrocytes from fixed tissues kept in different museum collections in Spain (EBD 8173) and Russia (specimens of *A. sturio*, *Acipenser nudiventris* Lovetzky, 1828, and *A. gueldenstaedtii*) should be taken with extreme caution and reservation: there is no guarantee that the sturgeon samples were fixed and then kept the same way in alcohol. For example, in Russian collections the alcohol-fixed specimens were usually transferred and kept for years in formalin, and then returned to alcohol. Since the Hernando *et al.* (1999a, b) publications cited do not contain any data on measurements and statistics, it is impossible to trust the authors' conclusion that "the average erythrocyte size in this fish (i.e., EBD-8173) did not differ significantly from that in Russian sturgeon *A. gueldenstaedtii* with a similar number of chromosomes (247-250) and was approximately 1.5-1.6 times larger than average erythrocytic size in the Baltic ($2n = 116 \pm 4$) and bastard sturgeon *A. nudiventris* ($2n = 118 \pm 2$)" (Hernando *et al.*, 1999b, p. 805) (co-editor's note; July 2000).

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